Correlating the Pharmacology and Molecular Biology of Opioid Receptors

Cloning and Antisense Mapping a Kappa$_3$-related Opiate Receptor[a]

G. W. PASTERNAK,[b–d] Y-X. PAN,[c] AND J. CHENG[c]

[c]*The Cotzias Laboratory of Neuro-Oncology*
Memorial Sloan-Kettering Cancer Center
and
[d]*Departments of Neurology & Neuroscience and Pharmacology*
Cornell University Medical College
New York, New York 10021

The pharmacology of the various opioid receptors has been well studied. The availability of large numbers of selective agonists and antagonists has permitted the correlation of specific receptors defined in binding assays with selected pharmacological actions (TABLE 1).[1,2] Virtually all the established opioid receptor subtypes elicit analgesia, although the localization of their actions varies. In addition to regional differences, highly selective agonists for the different subtypes do not demonstrate cross-tolerance, implying that they are activating distinct systems leading to a common response. A full understanding of these receptor mechanisms requires the elucidation of the molecular mechanisms responsible for these *in vivo* actions. We now review recent work from our laboratory correlating the molecular biology and pharmacology of opioid receptors.

DELTA RECEPTORS

Recently, two groups reported the cloning of the delta opioid receptor (DOR-1).[3,4] This seminal discovery was soon followed by the identification of clones encoding mu and kappa receptors.[5–14] All three families show high degrees of homology, but are quite distinct in both the N- and C-termini, as well as the second extracellular loop and the two adjacent transmembrane regions. When expressed, all three families show the anticipated binding selectivities towards large numbers of opioids. Functionally they are active, inhibiting stimulated adenylyl cyclase.

Although these studies all inferred that the cloned receptors corresponded to those identified pharmcologically, a formal connection had not been demonstrated.

[a]This work was supported by grants from the National Institute on Drug Abuse to G.W.P. (DA02615 and DA07242). G.W.P. is a recipient of a Research Scientist Award from the National Institute on Drug Abuse (DA00220), and Y-X.P. is supported by a fellowship from the Aaron Diamond Foundation.

[b]Address correspondence to Dr. Gavril W. Pasternak, Department of Neurology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

We addressed this question using an antisense approach to selectively down-regulate naturally occurring mRNA by using short oligodeoxynucleotides (ODN) with complementary sequences. We first examined delta receptors in a tissue culture model (FIG. 1).[15] We tested a series of antisense ODN directed at different regions of cDNA encoding the receptor, including both coding and untranslated regions. All the antisense ODN successfully down-regulated delta binding in the NG108-15 cells.[15] This indicated that the location of the antisense ODN on the mRNA was not critical. As controls, we also examined additional ODN. The sense ODN, which cannot anneal to the mRNA, was inactive. However, mixing the sense ODN with its corresponding antisense ODN prior to their addition to the cultured cells prevented the actions of the antisense ODN. A mismatch ODN, in which we scrambled the sequence of four bases without changing the overall base composition, also was without effect, demonstrating the stringent specificity of the response.

TABLE 1. Tentative Classification of Opioid Receptor Subtypes and Their Actions[a]

Receptor	Analgesia	Other
Mu		
Mu_1	Supraspinal	Prolactin release Feeding Acetylcholine release in the brain
Mu_2	Spinal	Respiratory depression Gastrointestinal transit Dopamine turnover in the brain Feeding Guinea-pig ileum bioassay Most cardiovascular effects
Kappa		
$Kappa_1$	Spinal	Diuresis Feeding
$Kappa_2$	Unknown	
$Kappa_3$	Supraspinal	
Delta		Mouse vas deferens bioassay
$Delta_1$	Supraspinal	
$Delta_2$	Spinal and supraspinal	

[a]Modified from Pasternak.[1,2]

We then examined antisense ODN *in vivo*. The paradigm employed injections of antisense on days 1, 3, and 5, followed by analgesic testing on day 6. We used this approach to obtain a prolonged down-regulation of receptor synthesis. The turnover of opioid receptors is approximately 3–4 days.[16] By maintaining the antisense treatments over 5 days, we permitted the loss of preexisting receptors. Administered intrathecally, the antisense selectively blocked the analgesic actions of two delta agonists, DPDPE and deltorphin II, without affecting either mu or delta actions (FIG. 2).[15] Again, the mismatch controls were without effect.

Additional controls explored the stability of the ODN under these conditions. In tissue culture studies, radiolabeled ODN is rapidly taken up by cells and as much as 5% remains associated with the cells as intact ODN, as indicated by its size on gels. Similar results were seen *in vivo*. Measurements of mRNA levels of DOR-1 following intrathecal administration reveal a 30% reduction, similar to the loss of binding.

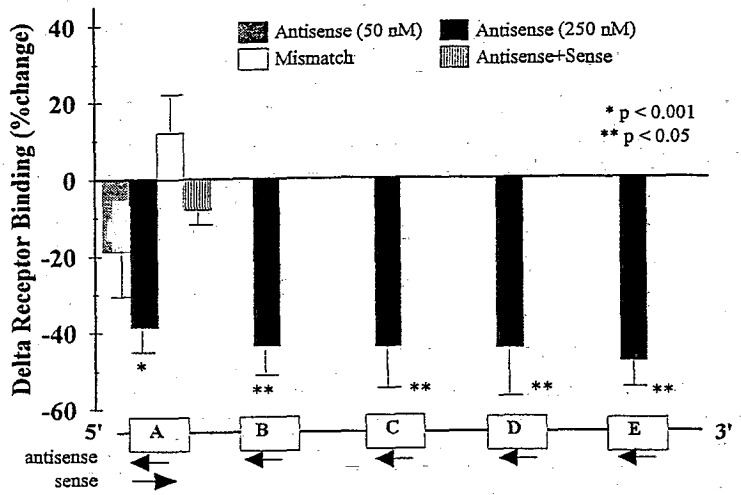

FIGURE 1. Down-regulation of delta receptor binding by antisense to DOR-1. NG108-15 cells were incubated for 5 days with the various antisense oligodeoxynucleotides at 250 nM, unless stated otherwise. The schematic representation provides an indication of the location of the antisense. Antisense A is directed at the N-terminus of the receptor, whereas the others are directed at regions downstream, including the 3'-untranslated region. (From Standifer *et al.*[15] Reproduced, with permission, from *Neuron*.)

Although the mechanism through which these ODN act remains unclear, they do down-regulate both receptors and mRNA.

MU AND KAPPA$_1$ RECEPTORS

Using similar approaches, we also demonstrated that antisense ODN directed against kappa$_1$[17] and mu receptors[18] also down-regulated the receptors against which they were designed. In all cases, our studies demonstrated remarkable selectivity among the various receptor classes, suggesting that this antisense approach might be a reasonable approach for selectively screening partial sequences of novel proteins for pharmacological activity without having to clone, sequence, and express complete gene products.

CLONING A KAPPA$_3$-RELATED OPIOID RECEPTOR

Several years ago, we identified a novel opioid binding site, termed kappa$_3$[19–23] (for review, see ref. 1). Although it fit the traditional criteria for inclusion in the opioid receptor family, it is readily distinguished from the mu, delta or kappa$_1$ receptors. Kappa$_3$ receptors display a binding profile that differs from the others and, in tissue culture studies, inhibits adenylyl cyclase through mechanisms independent of mu, delta or kappa$_1$ receptors.[24] *In vivo,* kappa$_3$ receptors elicit analgesia supraspi-

nally. Additionally, it demonstrates no cross-tolerance with the other subtypes, particularly mu receptors.[20,22]

The cloning of delta receptors quickly led to the identification of clones corresponding to the mu and kappa$_1$ receptors using reverse transcriptase-polymerase

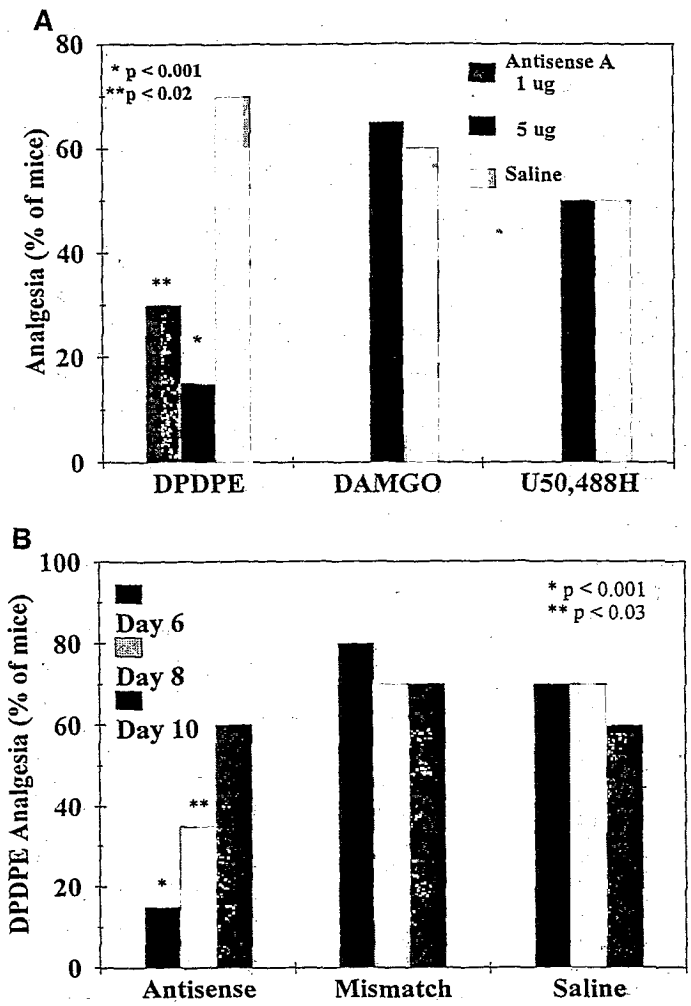

FIGURE 2. Selective blockade of delta analgesia by a DOR-1 antisense. (A) Groups of mice received Antisense A on days 1, 3, and 5 intrathecally, and analgesia was assessed on day 6 in the tailflick assay 15 min after receiving DPDPE (0.5 μg), DAMGO (8 ng) or U50,488H (25 μg). (B) Groups of mice received Antisense A, Mismatch A or saline on days 1, 3, and 5 and were tested for analgesia with DPDPE (0.5 μg) on days 6, 8, and 10. (From Standifer *et al.*[15] Reproduced, with permission, from *Neuron*.)

chain reaction. Anticipating strong homology among the opioid receptor classes, we employed a similar approach based upon the sequence of the delta receptors in an effort to look for additional opioid receptor subtypes. In addition to the sequences corresponding to delta, mu, and kappa$_1$ clones, we isolated a unique sequence of approximately 500 bases which had high homology to the others. Before committing our resources to cloning the full-length cDNA, we explored the pharmacological relevance of the PCR employing the antisense approach described above. We designed a 20 mer complimentary to the PCR product and administered it intracerebroventricularly (i.c.v.) in mice using the same paradigm we had previously developed for the other opioid receptors.[15,17,18] For a control, we designed a mismatch ODN in which the sequence of four bases of the 20 were scrambled, preventing effective annealing with the mRNA of interest. When we examined the animals, antisense treatment had little effect on the analgesic actions of morphine, DPDPE or U50,488, implying that the sequence was not encoding a mu, delta or kappa$_1$ receptor, respectively. However, the antisense effectively blocked the analgesic actions of the kappa$_3$ analgesic naloxone benzoylhydrazone (NalBzoH).[14,25] The mismatch control was inactive. This selective down-regulation of kappa$_3$ analgesia indicated that this clone was closely related to the kappa$_3$ receptor.

With this information, we proceeded to clone the full-length cDNA, which we termed KOR-3 (GenBank accession number U09421). The sequence is very similar to a recently reported putative opioid receptor, ORL1,[26] and has been observed by other groups.[14] Once we had obtained the full sequence, we expressed the clone in COS-7 cells. Using a monoclonal antibody (mAb8D8) directed against native kappa$_3$ receptors in BE(2)-C neuroblastoma cells, we found that on Western analysis the monoclonal antibody recognizes our clone expressed in COS-7 cells (Brooks, A., *et al.,* in preparation). Control COS-7 cells transfected with the vector lacking our clone did not display any immunoreactivity. The *in vitro* translation product of the clone also was recognized by Western using mAb8D8. Thus, both the antibody and antisense approaches closely associate the cloned receptor with the kappa$_3$ site. However, the expressed receptor was not functionally active. Attempts to demonstrate binding were ambiguous. Although we occasionally could see cyclase effects, they were not very robust.

In view of the difficulty in expressing a functional receptor, we returned to the antisense approach to determine whether the clone truly corresponds to the kappa$_3$ receptor. We chose five regions in the open reading frame as well as two regions in the 3'-untranslated region, designed antisense ODN, and performed antisense studies on analgesia. In all cases, the antisense blocked the kappa$_3$ analgesic response *in vivo.* Southern analysis indicates a single gene encoding the sequence, further strengthening the implication that the KOR-3 clone derives from the gene encoding the kappa$_3$ receptor. However, it does not explain the difficulty obtaining a functionally active receptor. A number of possibilities exist. Although the receptor is expressed in the COS-7 cells, needed posttranslational changes may not be taking place or necessary transduction systems or G-proteins may not be available. Alternatively, there may be splice variants of the receptor. Indeed, we have evidence for a splice site between the first and second transmembrane domains, a location similar to that seen with the other opioid receptor classes. Although we have not yet identified alternative sequences in the coding region, alternative splicing remains a strong possibility. All the antisense ODN directed downstream from the splice site down-regulated NalBzoH analgesia as noted above. However, when we examined an additional series of ODN directed at sequences upstream from the splice, only one of the five blocked NalBzoH analgesia. This response is quite distinct from that seen with the ODN designed against the regions downstream, all of which block kappa$_3$

analgesia. Although indirect, this may be an indication that the kappa$_3$ receptor is a splice variant of our clone, both being derived from a single gene.

SUMMARY

We cloned a kappa$_3$-related opioid receptor, and although it is still not clear whether this clone corresponds to the kappa$_3$ receptor itself or is a related gene product, the extensive antisense mapping and the antibody immunoreactivity strongly associate this clone with the kappa$_3$ receptor. Our approach also indicates the usefulness of antisense approaches in mapping and identifying orphan receptors. Perhaps it is most effective in identifying partial sequences prior to cloning them in their entirety. It also provides a mechanism of identifying proteins that are not expressed functionally.

REFERENCES

1. PASTERNAK, G. W. 1993. Clin. Neuropharmacol. **16:** 1–18.
2. PASTERNAK, G. W. 1988. J. Am. Med. Assoc. **259:** 1362–1367.
3. EVANS, C., D. KEITH, H. MORRISON, K. MAGENDZO & R. EDWARDS. 1992. Science **258:** 1952–1955.
4. KIEFFER, B., K. BEFORT, C. GARERIAUX-RUFF & C. HIRTH. 1992. Proc. Natl. Acad. Sci. USA **89:** 12048–12052.
5. CHEN, Y., A. MESTEK, J. LIU, A. HURLEY & L. YU. 1993. Mol. Pharmacol. **44:** 8–12.
6. FUKUDA, K. S. KATTO, K. MORI, M. NISHI & H. TAKESHIMA. 1993. FEBS Lett. **327:** 311–314.
7. MENG, F., G. XIE, R. THOMPSON, A. MANSOUR, A. GOLDSTEIN, S. WATSON, & H. AKIL. 1993. Proc. Natl. Acad. Sci. USA **90:** 9954–9958.
8. MINAMI, M., T. TOYA, Y. KATAO, K. MAEKAWA, S. NAKAMURA, T. ONOGI, S. KANEKO & M. SATOH. 1993. FEBS Lett. **329:** 291–295.
9. TAKESHIMA, H., K. FUKUDA, S. KATO & K. MORI. 1993. FEBS Lett. **330:** 77–80.
10. THOMPSON, R. C., A. MANSOUR, H. AKIL & S. J. WATSON. 1993. Neuron **11:** 1–20.
11. WANG, J.-B., Y. MEI, C. M. EPPLER, P. GREGOR, C. E. SPIVAK & G. R. UHL. 1993. Proc. Natl. Acad. Sci. USA **90:** 10230–10234.
12. YASUDA, K., K. RAYNOR, H. KONG, C. BREDER, J. TAKEDA, T. REISINE & G. BELL. 1993. Proc. Natl. Acad. Sci. USA **90:** 6736–6740.
13. REISINE, T. & G. BELL. 1993. Molecular biology of opioid receptors. Trends Neurosci. **16:** 506–510.
14. UHL, G. R., S. R. CHILDERS & G. W. PASTERNAK. 1994. Trends Neurosci. **17:** 89–93.
15. STANDIFER, K. M., C.-C. CHIEN, C. WAHLESTEDT, G. P. BROWN & G. W. PASTERNAK. 1994. Neuron **12:** 805–810.
16. PASTERNAK, G. W., S. R. CHILDERS & S. H. SNYDER. 1980. J. Pharmacol. Exp. Ther. **214:** 455–462.
17. CHIEN, C. C., G. P. BROWN, Y. X. PAN & G. W. PASTERNAK. 1994. Eur. J. Pharmacol. **253:** R7–8.
18. ROSSI, G., Y. X. PAN, J. CHENG & G. W. PASTERNAK. 1994. Life Sci. **54:** PL375–379.
19. PRICE, M., M. A. GISTRAK, Y. ITZHAK, E. F. HAHN & G. W. PASTERNAK. 1989. Mol. Pharmacol. **35:** 67–74.
20. GISTRAK, M. A., D. PAUL, E. F. HAHN & G. W. PASTERNAK. 1989. J. Pharmacol. Exp. Ther. **251:** 469–476.
21. CLARK, J. A., L. LIU, M. PRICE, B. HERSH, M. EDELSON & G. W. PASTERNAK. 1989. J. Pharmacol. Exp. Ther. **251:** 461–468.
22. PAUL, D., J. A. LEVISON, D. H. HOWARD, C. G. PICK, E. F. HAHN & G. W. PASTERNAK. 1990. J. Pharmacol. Exp. Ther. **255:** 769–774.

23. PAUL, D., C. G. PICK, L. A. TIVE & G. W. PASTERNAK. 1991. J. Pharmacol. Exp. Ther. **257:** 1–7.
24. STANDIFER, K. M., J. CHENG, A. BROOKS, W. SU, C. HONRADO & G. W. PASTERNAK. 1994. J. Pharmacol. Exp. Ther. **270:** 1246–1255.
25. PAN, Y. X., J. CHENG & G. W. PASTERNAK. 1994. Regul. Pept. **54:** 217–218.
26. MOLLEREAU, C., M. PARMENTIER, P. MAILLEUX, J. L. BUTOUR, C. MOISAND, P. CHALON, D. CAPUT, G. VASSART & J. C. MEUNIER. 1994. FEBS Lett. **341:** 33–38.